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during Egg Activation in the Annelid, *Chaetopterus*

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Transient waves of Ca^{2+} release cross-fertilizing deuterostome eggs from the point of sperm entry to its antipode and provide much of the activating stimulus for the egg. Based on several indirect lines of experimental evidence, it was proposed that protostome eggs are activated by a prolonged uptake of Ca^{2+} from the medium due to sperm-induced membrane depolarization and that this uptake then starts an activation wave similar to those in deuterostomes, except that it moves inward from the whole surface rather than through the egg from pole to pole. To test these hypotheses, we microinjected oocytes of the polychaete annelid, *Chaetopterus pergamentaceus,* with semisynthetic recombinant aequorins and measured light emission in response to both fertilization and artificial activation by excess K^+. Both fertilization and K^+-activation induced multiple, brief Ca^{2+} transients in the eggs. The first transient did not propagate, but it was followed by a series of globally propagated Ca^{2+} waves interspersed with additional nonpropagated pulses. The waves traversed the egg at about 30 μm/sec. Sequential propagated waves and nonpropagated pulses generally originated at different regions on the egg surface, except the last few, which originated in the same "pacemaker" region. These new data are consistent with the hypothesis that the activation of protostome eggs is initiated by Ca^{2+} waves. However, the fact that these waves propagated from pole to pole like those in deuterostome eggs refutes the notion that Ca^{2+} waves in activating protostome eggs move inward from the whole surface.

INTRODUCTION

Transient increases in free cytoplasmic Ca^{2+} pass through the cytosol of a wide variety of fertilizing deuterostome eggs and do so from the sperm entry point to its antipode. These Ca^{2+} waves occur through the release of stored Ca^{2+}, and they provide most or all of the activating stimulus for these eggs (Jaffe, 1983b, 1985; Whitaker and Steinhardt, 1985). It is unclear how these waves are initiated. They may be initiated by a localized Ca^{2+} release due to synthesis of inositol (1,4,5)-trisphosphate ($InsP_3$) or cyclic ADP ribose (cADPR) (Dargie *et al.*, 1990; Galione *et al.*, 1991, 1993) or to a localized Ca^{2+} release due to Ca^{2+} that enters the egg from the sperm or from the medium (Jaffe, 1991). In either case, once initiated, the waves may be propagated by the elevated Ca^{2+} itself, i.e., by Ca^{2+}-induced Ca^{2+} release (CICR) or by Ca^{2+}-sensitized $InsP_3$-induced Ca^{2+} release (Miyazaki *et al.*, 1992).

Ca^{2+} waves have been imaged in the eggs of several deuterostomes, including those of the sea urchin, ascidian, fish, frog, and mammal. In sea urchins, there is an initial, small Ca^{2+} rise evidently due to membrane depolarization, followed by a single wave of $InsP_3$-induced Ca^{2+} release (Gillot and Whitaker, 1993; Shen and Buck, 1993). This wave is followed by another, associated with pronuclear movement, and a third transient associated with pronuclear fusion (Gillot and Whitaker, 1994). Ascidian (Speksnijder *et al.*, 1989, 1990; Speksnijder, 1992) and mammalian (Miyazaki *et al.*, 1986, 1993; Kline and Kline, 1992; Fissore and Robl, 1994) eggs undergo a series of Ca^{2+} waves. Fish and frog eggs have been reported to undergo a single wave of Ca^{2+} release (Gilkey *et al.*, 1978; Busa *et al.*, 1985). In frog eggs, this Ca^{2+} release requires $InsP_3$ synthesis (Nuccitelli *et al.*, 1993). In sea urchin egg homogenates both cADPR and $InsP_3$ can induce Ca^{2+} release, whereas in frog homogenates $InsP_3$ can, but cADPR cannot (Galione *et al.*, 1993). In mammalian eggs, $InsP_3$ appears to be the primary factor in eliciting Ca^{2+} release (Miyazaki *et al.*, 1992, 1993; Fissore and Robl, 1994; Xu *et al.*, 1994).

The activation program of protostome eggs differs somewhat from that of deuterostomes. Unlike deuterostome eggs, those of protostomes can be activated artificially by treatment with excess K^+ (Loeb, 1901; Allen, 1953), which

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depolarizes the egg plasma membrane. Depolarization of deuterostome (sea urchin) eggs provides the rapid block to polyspermy and permits some Ca^{2+} entry (Gillot and Whitaker, 1993), but it does not activate the eggs (Jaffe and Cross, 1986). Depolarization also evidently provides a rapid block to polyspermy in eggs of the polychaete annelid, *Chaetopterus pergamentaceus* (Jaffe, 1983a; Eckberg and Anderson, 1985).

Based on several indirect lines of experimental evidence, it was proposed that protostome eggs are activated by a prolonged uptake of Ca^{2+} from the medium due to sperm-induced membrane depolarization and that this uptake then starts an activation wave similar to those in deuterostomes, except that it moves inward to the center from the whole surface, rather than across the egg from pole to pole (Jaffe, 1983b, 1985, 1991). To test this hypothesis, we microinjected unfertilized, metaphase I-arrested *Chaetopterus* oocytes with semisynthetic recombinant aequorin (Shimomura *et al.*, 1993) and imaged light emission in response to both fertilization and artificial activation by excess K^+. If Ca^{2+} uptake caused by sperm-induced membrane depolarization is the mechanism by which these eggs are activated to develop, eggs activated by sperm and excess K^+ should show similar Ca^{2+} responses. The results showed that both fertilization and K^+-activation induced similar, multiple, brief Ca^{2+} transients in the eggs. These responses included both propagated fast waves and nonpropagated localized pulses of Ca^{2+} release. However, these waves propagated from pole to pole like those of deuterostome eggs, disproving the notion that such waves move inward radially. A short, preliminary report of some of the effects of sperm activation on Ca^{2+} waves has appeared previously (Eckberg *et al.*, 1993).

MATERIALS AND METHODS

Gametes and Microinjection

Adult specimens of *Chaetopterus pergamentaceus* were obtained from the Marine Resources Department, Marine Biological Laboratory (Woods Hole, MA). Females were rinsed with distilled water to kill any adsorbed sperm, and metaphase I-arrested oocytes were obtained by teasing the eggs out into filtered natural sea water and washing them three to four times. They were then placed in Ca^{2+}-free MBL-formula artificial sea water for microinjection.

We used aequorin luminescence to detect intracellular Ca^{2+} (Blinks, 1989; Shimomura *et al.*, 1993; Miller *et al.*, 1994). Aequorin has several attributes that make it advantageous for these studies. First, it is nontoxic, and its detection does not require that the cells be UV-irradiated. Second, it has negligible Ca^{2+} buffering capacity at the intracellular concentrations used. Third, it has a wide dynamic range, and its sensitivity has been increased by combining the apoaequorin with synthetic coelenterazine analogs (Shimomura *et al.*, 1993). Fourth, it gives extremely low background signals because the eggs are not autoluminescent. Fifth, since light emission increases exponentially with increasing Ca^{2+} (at least over physiologically significant Ca^{2+} concentrations), aequorin gives inherently high contrast. Finally, aequorin remains in the cytosol for very long periods and does not partition into cytoplasmic organelles (Miller *et al.*, 1994).

Eggs were microinjected by the modified Hiramoto/Kiehart method, details of which have been published elsewhere (Miller *et al.*, 1994). In the experiments reported, we used semisynthetic recombinant *h* and *f*-aequorins (Shimomura *et al.*, 1993). These species of aequorin have a useful dynamic range of 0.01–10 μM Ca^{2+} and a sensitivity some 20-fold greater than that of native aequorin. Methods and precautions for handling and microinjecting such ultrasensitive semisynthetic recombinant aequorins have also been published (Shimomura, 1991; Miller *et al.*, 1994).

Luminescent Imaging

Our imaging system utilized a Zeiss Axiovert 100TV microscope coupled to a resistive anode-based imaging photon detector (IPD; Phototek, Ltd., St. Leonard-on-Sea, East Sussex, UK) and a video camera for the intermittent capture of bright-field images. IPD and video images were obtained through a 63X, 1.25 NA PLAN-NEOFLUAR lens. Other details of the imaging system have been recently described. This system identified each photon captured by its *x*, *y* coordinates and time. Images were then analyzed using software developed in the Calcium Imaging Laboratory at the MBL, which allowed the data to be played back in numerous formats and to be analyzed quantitatively (Miller *et al.*, 1994).

In some experiments on K^+-activated eggs, an identical optical platform and pathway was used with a Hamamatsu C-2400-47 ultra high sensitivity intensified camera (Hamamatsu Corp., Bridgewater, NJ) instead of the IPD. Output was fed from the camera to a Hamamatsu Argus-10 image processor and stored as grayscale images in NTSC format on videotape. Individual frames were digitized and colorized using a Macintosh Quadra 660AV and the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, VA, part no. PB93-504868). In this experimental system, photons were accumulated continuously throughout each individual wave of Ca^{2+}. This instrument configuration and experimental protocol permitted the two-dimensional visualization of Ca^{2+} increases, but did not permit us to observe the resequestration of Ca^{2+} (decay of luminescence) or to play back the waves in different time formats. However, it did allow us to detect Ca^{2+} transients in two dimensions. Similar waves of Ca^{2+} release were observed with both detector types.

Intracellular Distribution of Labeled Aequorin

We observed uniform, weak luminescence from aequorin-injected oocytes that were not exposed to sperm or to excess

K^+, suggesting that the injected aequorin diffuses throughout the cytosol. To determine the cellular distribution of microinjected aequorin in the eggs more directly, fluorescein-conjugated aequorin (Shimomura *et al.,* 1993) was injected and the eggs were examined by confocal fluorescence microscopy using a Zeiss LSM 410 Axiovert at the Zeiss Microscopy Laboratory at the MBL. Images were obtained using a 40X 0.6 NA ACHROPLAN lens and Zeiss LSM software. Other than an initial adjustment of brightness and contrast, the images were not further processed.

Images of optical sections through the hemisphere of the egg that was close to the objective lens showed uniform fluorescence, demonstrating that aequorin was present equally throughout the cytoplasm, images of sections near the equator appeared to show more intense fluorescence in the cortex than in the endoplasm, and images through the hemisphere away from the objective lens exhibited very little fluorescence, except in the region of the spindle in specimens in which the spindle was in this position relative to the light path (Fig. 1). This suggests that the opaque endoplasmic granules (mostly yolk and lipid) prevent the fluorescence from labeled aequorin deep in the cytoplasm from reaching the objective lens, probably due to their absorbing out the excitatory and/or emitted light. The observation of fluorescence in the spindle (Fig. 1d), which lacks the large endoplasmic granules, supports the conclusion that these granules are responsible for most of the absorption. Whichever components are responsible for the diminished signal, we conclude that microinjected aequorin freely diffuses throughout the cytosol of the *Chaetopterus* egg.

Fertilization

Before inseminating the eggs, we observed them with the IPD-based imaging system to ensure that they had not been damaged by the injection. After we observed a stable baseline luminescence for at least 10 min, the eggs were inseminated by replacing some of the sea water in the Hiramoto chamber with sea water containing motile sperm. Sea water can be changed in the outer part of the chamber by a Pasteur pipet without disturbing the eggs under their supporting wedge. This procedure has the advantage that, since the medium in direct contact with the eggs is not replaced, some time is required for test chemicals to diffuse or sperm to swim to the eggs, allowing all the eggs to be placed back in the imaging system before they become fertilized or artificially activated. Since there is no cortical reaction in *Chaetopterus* eggs, the criterion we used for egg activation was polar body formation, which occurs 10–15 min after insemination. Injected and uninjected (control) eggs formed polar bodies at the same time.

K^+-Activation

Eggs were K^+-activated by exposing them to natural sea water (NSW) containing excess K^+, made by adding 2.5 *M* KCl to the NSW. Except where otherwise noted, KCl was added to a final concentration of 100 m*M*. This concentration was derived empirically (see below). After the baseline luminescence was recorded, the NSW in the chamber was replaced with NSW containing excess K^+. The eggs were exposed to high K^+ throughout the subsequent recording, which lasted up to 30 min. These eggs formed polar bodies and/or became ameboid (Lillie, 1902). Eight K^+-activated eggs were examined, four using the IPD and four using the Hamamatsu system.

RESULTS

Fertilization-Induced Ca^{2+} Fluxes

To determine the time course of any Ca^{2+} transients after fertilization, we counted photons as a function of time after insemination. Fertilization induced multiple, brief Ca^{2+} transients in the eggs over a period of 7–10 min. Figure 2 shows the amplitude and duration of the sequence of transients in a typical fertilized egg. This egg underwent 9 resolved transients during 7.5 min. After that, the Ca^{2+} transients ceased. These transients are not associated with the meiotic divisions, because the latter do not begin until approximately 10 min after fertilization. The durations of individual transients were very consistent. Most were maximal within 10 sec and lasted no longer than 20 sec. The number and duration of Ca^{2+} transients shown here is typical; half of the eggs studied exhibited either 9 or 10 transients. However, we have observed as few as 3 and as many as 27 transients from a single normal egg (Table 1). The intervals between transients, though they varied significantly between eggs, were quite regular within a single egg (cf. Fig. 2). In this egg, the mean time (±SD) between Ca^{2+} peaks was 0.80 ± 0.16 min. Similar results were obtained from eight other eggs which did not form polar bodies, presumably because they were compressed in the Hiramoto chamber.

After the sequence of transients, the aequorin luminescence returned to basal levels; thereafter, we observed no further transients. In four experiments, we then lysed the zygotes by perfusing sea water containing 2% Triton X-100 into the chamber. In each case, lysis was accompanied by extremely intense luminescence throughout the egg, demonstrating that the aequorin had not been exhausted during the experiment. Therefore, the return of luminescence to basal levels following the series of transients indicates a real return of Ca^{2+} to a physiological resting level.

To estimate the extent of the Ca^{2+} rise during a transient, we had to make two assumptions. First, we assumed that *Chaetopterus* oocytes, like other unfertilized marine eggs, have a resting free Ca^{2+} concentration of approximately 100 n*M* (Eisen and Reynolds, 1985; Poenie *et al.,* 1985; Whitaker and Patel, 1990; Stricker *et al.,* 1992). Second, we assumed that the luminescence of semisynthetic recombinant aequorins varies with Ca^{2+} concentration *in vivo* as it does *in vitro* (Shimomura *et al.,* 1993). Given these two assumptions, the peak photon levels indicate peak Ca^{2+} levels of approximately 400–600 n*M*. This is somewhat less than

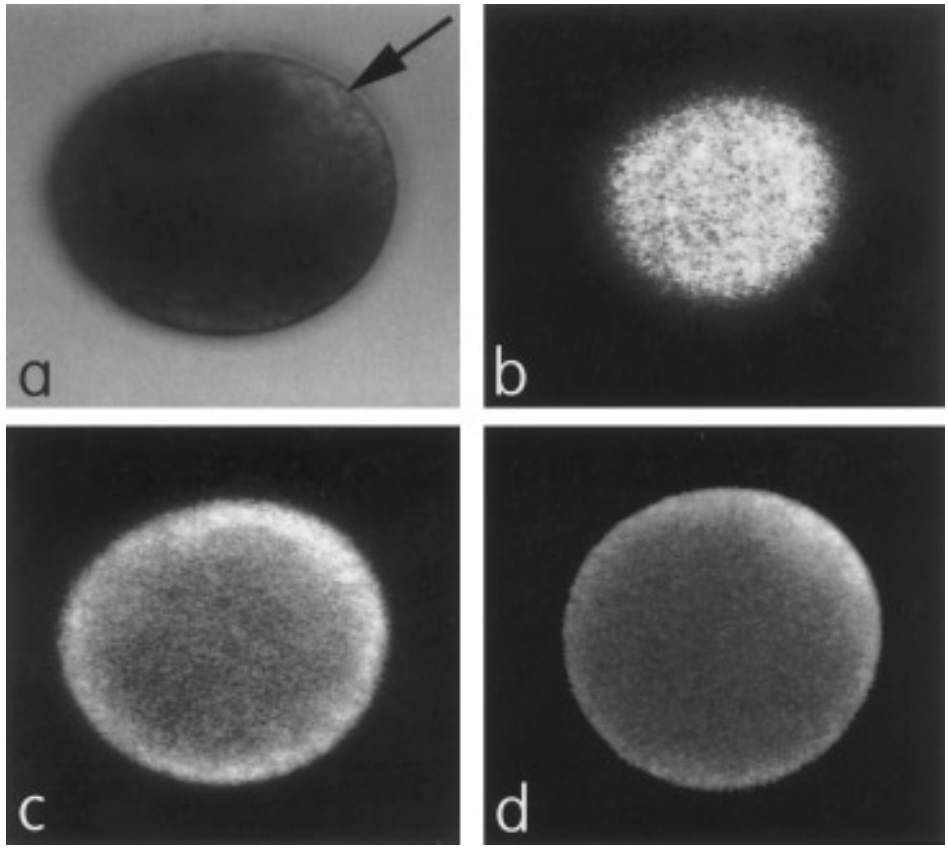


FIG. 1. Aequorin distribution in a *Chaetopterus* egg that was microinjected with fluorescein-tagged aequorin. The egg was compressed to about 65 μm thick, or approximately two-thirds of its spherical diameter. (a) Bright-field light micrograph of the egg. The arrow indicates the position of the meiotic spindle. (b–d) Confocal fluorescence images. (b) A section 15–20 μm into the egg. Note the uniform fluorescence throughout the cytosol. (c) A section 25–30 μm into the egg. The fluorescence appears to be restricted to the periphery because the light is absorbed by components of the opaque endoplasm. (d) A section 45–50 μm into the egg. Fluorescence appears to be restricted to the area of the spindle, because of the absorption of light by the opaque endoplasm elsewhere. The spindle lacks the opaque granular elements seen elsewhere in the endoplasm.

the levels achieved during the single peak in fertilizing sea urchin eggs (Poenie *et al.*, 1985), but is comparable to that in fertilized mouse eggs (Miyazaki *et al.*, 1992).

To analyze these transients further, we examined the spatial distribution of photons in the egg as a function of time. Generally, the first transient was localized to one region of the egg; such a transient is shown in Fig. 3 (pulse A in Fig. 2). This was followed by one or more global waves of Ca^{2+} release. Figure 4 shows the first propagated wave in this egg (pulse B in Fig. 2). Typically, several such waves were observed sequentially in an egg (Table 1). The waves traversed the egg at about 30 μm/sec. Additional nonpropagated pulses were interspersed with the waves.

Figure 5 summarizes the spatial distribution of the waves and nonpropagated pulses resolved in the egg examined in Figure 2. All but one of them clearly originated in the cortex. Waves originated from all regions of the egg surface, and sequential waves and pulses generally originated at different regions of the egg surface. However, the last few (e.g., G–I in Fig. 5) originated from the same region. By analogy with work previously done on ascidian eggs (Speksnijder *et al.*, 1989, 1990; Speksnijder, 1992), we will identify this region as a ''pacemaker.'' Every egg studied established such a pacemaker region (Table 1). Although most of the waves appeared to traverse the deeper parts of the cell, confocal or 3-D luminsescent imaging would be necessary to establish this unambiguously. Some, such as the wave in Fig. 5C, could be clearly seen to propagate exclusively through the cortex.

Figure 6 summarizes the appearances of the initial pulses in each of the eggs described in Table 1. Some of these transients (Figs. 6B, 6C, and 6E) never extended beyond their initial boundaries; others (Figs. 6A, 6D, and 6F) appeared to begin to propagate, but failed to do so.

K^+-Induced Ca^{2+} Fluxes

To examine the effect of K^+-activation on the Ca^{2+} transients, we first determined the optimal K^+-concentration to

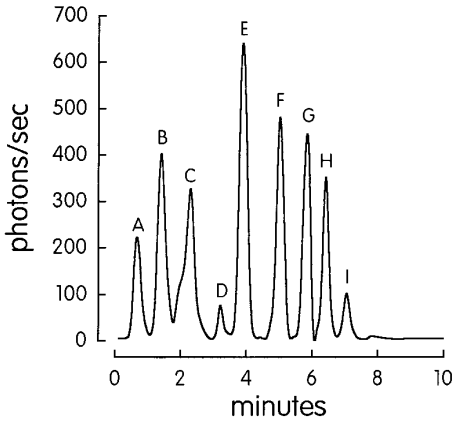


FIG. 2. The sequence of Ca^{2+} transients in a *Chaetopterus* egg microinjected with semisynthetic *f*-recombinant aequorin and then fertilized. Photon counts/sec were averaged over successive 7.7-sec intervals and plotted. This egg underwent nine Ca^{2+} transients over a $7\frac{1}{2}$-min period after fertilization. The average periodicity of the transients was 0.80 ± 0.16 min. The letters A–I identify the transients examined in subsequent figures.

add to the eggs. K^+-activation of *Chaetopterus* eggs has been known for many years (Loeb, 1901; Lillie, 1902), but there has been no systematic quantitative study on the effects of excess K^+ on the activation of these eggs. Furthermore, whereas previous studies have primarily examined later developmental events (differentiation without cleavage), we used polar body formation and the initiation of ameboid activity as our criteria of activation. Figure 7A shows the dose-dependent activation of *Chaetopterus* eggs by these criteria in response to excess K^+. Activation was maximal above a 50 m*M* K^+ excess. Excess Na^+ was ineffective at concentrations up to fourfold higher. This confirms Loeb's conclusions and suggests that K^+ probably acts by depolarizing the cell membrane. It does not activate eggs by increasing tonicity.

To be certain that these concentrations of K^+ were not injurious to the eggs, we exposed fertilized eggs to them beginning 3 min after insemination. Continuous exposure to either excess K^+ or Na^+ inhibited cleavage in a dose-dependent manner with approximately 100 m*M* excess K^+ or Na^+ giving 50% inhibition of cleavage. However, an exposure to either ion for 20 min, the duration used to activate eggs, had no effect on the ability of fertilized eggs to cleave (Fig. 7B). These results suggest that the inhibitory effect of the ions on cleavage is due to the hypertonicity of the sea water and is, therefore, unrelated to the activating effect. We selected a 100 m*M* K^+ excess as the standard activating concentration, because it was slightly higher than the minimum concentration for a full response but was not inhibitory to normal development.

K^+-activation also induced a series of Ca^{2+} transients (Fig. 8), including sequential waves of Ca^{2+} release. These waves were very similar in velocity to those observed in fertilized eggs, but the peak Ca^{2+} levels reached were slightly lower (approximately 300 n*M*), and the final resting level of Ca^{2+} in K^+-activated eggs was higher than that in fertilized eggs. If these eggs were subsequently returned to NSW, this higher resting level of Ca^{2+} returned to normal.

Like fertilized eggs, such eggs first exhibited a nonpropagated pulse of Ca^{2+}. An example of such a pulse obtained by the Hamamatsu intensified camera is shown in Fig. 9. Though this pulse is restricted to the top of the egg, the figure also shows a less intense Ca^{2+} rise throughout the cortex. Such increased luminescence throughout the cell cortex generally accompanied Ca^{2+} transients in K^+-activated eggs, but its level was much lower than that observed in the waves and nonpropagated pulses.

The first wave in this egg is shown in Fig. 10. This wave took approximately 6 sec to traverse the egg. Because sampling with this apparatus accumulated photons throughout a wave, the return to background could not be observed, and the color scale is only semiquantitative. However, even with these limitations, the wave propagation in K^+-activated eggs was indistinguishable from that in fertilized eggs.

DISCUSSION

It is well established that deuterostome development is initiated by Ca^{2+} waves that begin at the point where the

TABLE 1

Summary of Data Obtained from Six Fertilized Eggs on the Sequential Appearance of Propagated and Nonpropagated Pulses

Egg no.	First transient	No. of transients	No. propagated	Initiation points vary?	Pacemaker established?
A	Nonpropagated	9	7	Yes	Yes
B	Nonpropagated	10	4	Yes	Yes
C	Nonpropagated	3	2	No	Yes
D	Nonpropagated	27	20	Yes	Yes
E	Nonpropagated	5	3	ND	ND
F	Nonpropagated	10	8	Yes	Yes

Note. ND, it could not be determined for certain whether the initiation points varied or whether a pacemaker was established, because the egg moved in the chamber.

fertilizing sperm fuses with the egg and traverse the egg. It has been hypothesized that protostome development is also initiated by Ca^{2+} waves through the egg, but that these waves begin at the whole surface and move inward to the center (Jaffe, 1983b, 1985, 1991). The results of previous studies have shown that the intracellular free Ca^{2+} concentration of protostome eggs increases upon fertilization (Deguchi and Osanai, 1994) or ionic activation (Wang *et al.*, 1991; Lindsay *et al.*, 1992). The new data presented herein are the first to image waves of free Ca^{2+} as a fertilization response in protostome eggs. These data are therefore consistent with the hypothesis that the activation of protostome eggs is initiated by Ca^{2+} waves, but the notion that such waves move inward radially from the whole surface is clearly incorrect. All the waves we have seen in *Chaetopterus* moved from pole to pole like those in deuterostome eggs. We did not determine the point of sperm entry in any of these waves. However, since sequential waves and nonpropagated pulses initiated at different points on the egg surface, we can conclude that many of the waves did not originate at the point of sperm entry. The rapidity with which the pulses and waves were extinguished indicates that the eggs have powerful mechanisms with which to pump Ca^{2+} out of the cytosol almost immediately after it is released.

The facts that sequential waves and nonpropagated pulses originated from different regions of the egg cortex and the last few waves originated from a common area suggest that these Ca^{2+} waves may set up a pacemaker region as has been reported in ascidian zygotes (Speksnijder *et al.*, 1989, 1990; Speksnijder, 1992). Such a pacemaker could either reflect preexisting polarity or be involved in the subsequent establishment of embryonic polarity. However, the waves in *Chaetopterus* zygotes differ from those of ascidians in their timing. The waves in ascidians occurred throughout the meiotic divisions, whereas those we measured in *Chaetopterus* were completed before the first meiotic division.

It is intriguing that some of the transients propagated across the egg, whereas others in the same egg did not. The rise time and duration of transients that did and did not propagate were similar. However, the peak Ca^{2+} levels in propagating waves were slightly higher than in those that did not propagate (compare the photon densities in Figs. 3 and 8 with those in Figs. 4 and 9). This suggests that a threshold Ca^{2+} concentration might have to be reached rapidly before a transient propagates (Fabiato, 1985; Carroll *et al.*, 1994).

That similar waves and nonpropagated pulses of Ca^{2+} release can be initiated by excess K^+ is another new finding in this study. Excess extracellular K^+ depolarizes the plasma membrane, resulting in voltage-gated Ca^{2+} entry. Interestingly, small, simultaneous Ca^{2+} increases throughout the entire cortex were associated with the localized or propagated transients of higher Ca^{2+} concentration. These most likely represent voltage-gated Ca^{2+} uptake, as recently reported for sea urchin eggs (Gillot and Whitaker, 1993; Shen and Buck, 1993) and spontaneously maturing mouse oocytes (Carroll *et al.*, 1994). As in fertilized eggs, pulses that did not propagate had lower peak intensities than those that propagated. Presumably, the global cortical rise is inhomogeneous, with local ''hot spots'' achieving a sufficient Ca^{2+} concentration to elicit a localized Ca^{2+} release; if the Ca^{2+} rise is sufficient and rapid enough, the localized release propagates across the cell.

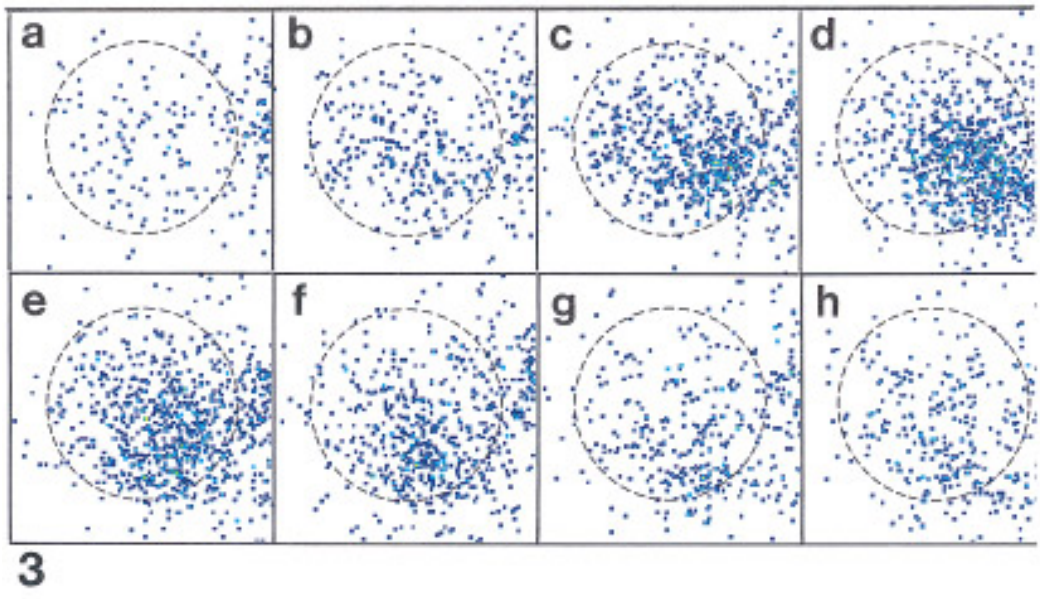
A Ca^{2+} wave was also reported in response to Mg^{2+}-activation of shrimp eggs (Lindsay *et al.*, 1992). The mechanism by which Mg^{2+} elicits this response is unclear, but it may involve a tyrosine kinase and/or InsP3 production (Lindsay and Clark, 1994).

The voltage-gated Ca^{2+} entry imaged in these studies has been proposed to be solely responsible for increasing cytoplasmic free Ca^{2+} after fertilization in protostome eggs (Jaffe, 1983b, 1985). K^+-activation is dependent on extracellular Ca^{2+} in protostomes, as removal of extracellular Ca^{2+} blocks the intracellular Ca^{2+} responses to K^+-activation (Deguchi and Osanai, 1994) and also blocks subsequent development (Ikegami *et al.*, 1976; Dubé and Guerrier, 1982). Consequently, we expected that excess extracellular K^+ would elicit a detectable increase in cortical Ca^{2+}, and we observed such increases.

The egg's plasma membrane should remain depolarized throughout exposure to excess K^+. If the resulting voltage-gated Ca^{2+} uptake were the sole mechanism by which excess K^+ increases intracellular Ca^{2+}, the intracellular Ca^{2+} should have been elevated continuously, and we should not have observed either the waves or the localized pulses of elevated intracellular Ca^{2+}. Therefore, the fact that sequential waves of Ca^{2+} release similar to those observed in fertilized eggs were also observed in K^+-activated eggs refutes this hypothesis and supports the alternative hypothesis that membrane depolarization initiates a series of intracellular Ca^{2+} releases.

Results obtained by others using mollusc eggs also support this model. Removal of extracellular Ca^{2+} does not block Ca^{2+} transients after fertilization (Wang *et al.*, 1991; Deguchi and Osanai, 1994). This would not be expected if the postfertilization Ca^{2+} transients were dependent upon voltage-gated Ca^{2+} uptake. The fact that Ca^{2+} transients in K^+-activated, but not in fertilized eggs, are dependent upon extracellular Ca^{2+} indicates that, despite the similarity of the responses, excess K^+ does not induce the Ca^{2+} release by the same mechanism(s) as does fertilization. Taken together, these results provide strong support for our previous conclusion (Bloom *et al.*, 1988) that excess K^+ does not fully mimic the effects of sperm in activating protostome eggs.

A technical limitation in this study is that photon detection by the IPD and intensified camera used permits spatial resolution only in two dimensions. Consequently, data resulting from waves initiated approximately perpendicular to the plane of focus could appear to represent uniform, overall increases in Ca^{2+}. We consider it to be improbable that the few such increases we observed represent real uniform overall increases, because (1) a large majority of the Ca^{2+} transients we observed either clearly passed through the cytoplasm in waves or else were localized exclusively within the cortex, and (2) no biochemical mechanisms are



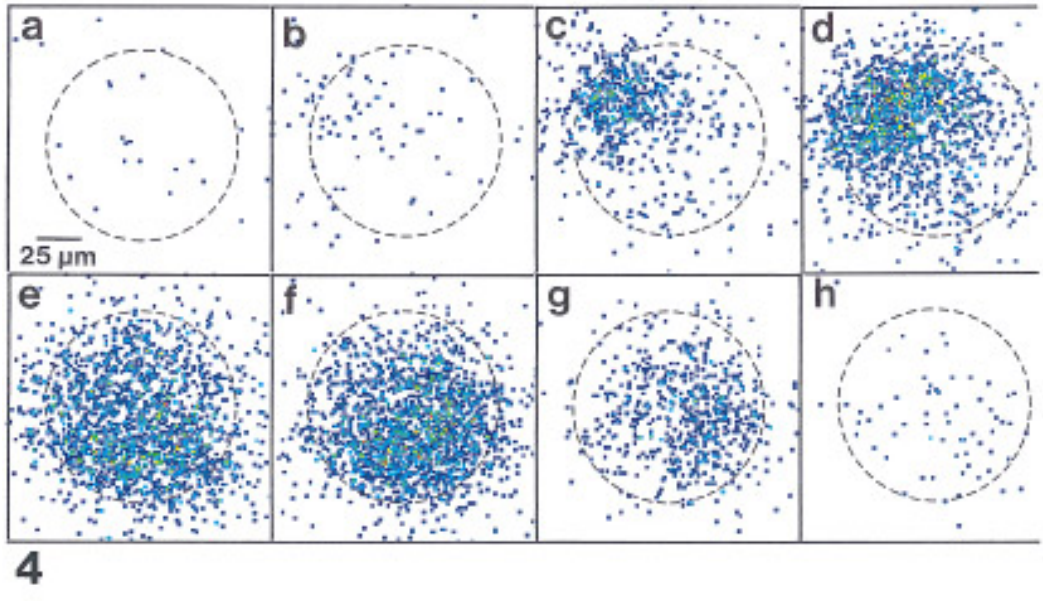
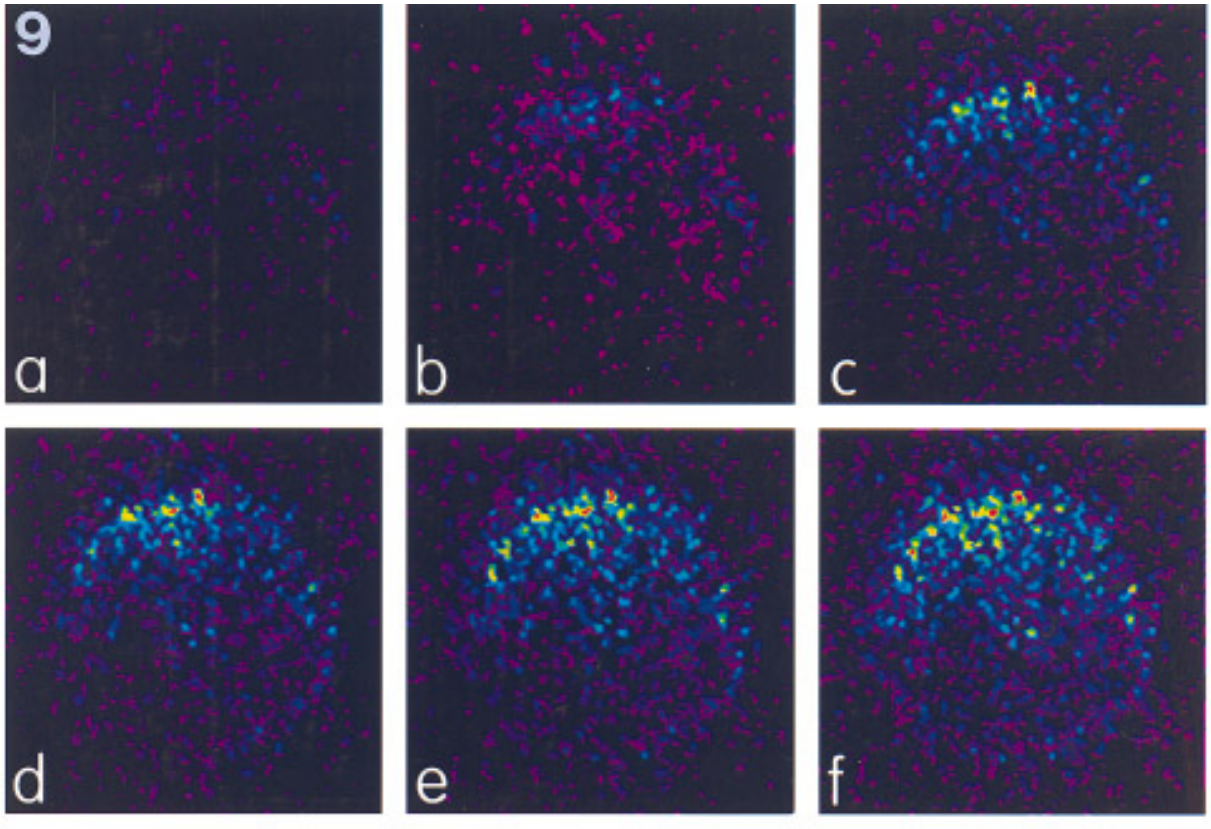


FIG. 3. An example of the first Ca^{2+} transient in a fertilized *Chaetopterus* egg. (A–H) The photons accumulated during successive 3-sec intervals. (A and H) The resting levels before and after the transient, which lasted approximately 9 sec.

FIG. 4. An example of a wave of Ca^{2+} release in a fertilized *Chaetopterus* egg. (A–H) The photons accumulated during successive 3-sec intervals. This wave moves from the upper left to the lower right. (A and H) The resting levels before and after the wave, which took approximately 9 sec to traverse the egg. The egg was injected with *f*-recombinant aequorin prior to insemination. This was the first wave in this egg, but subsequent waves were similar.

FIG. 9. An example of a nonpropagated Ca^{2+} release in a KCl-activated egg. The transient lasted approximately 4 sec. (a–f) The photons accumulated after successive 2-sec intervals. These data were obtained using the Hamamatsu system, so the photons shown in successive panels are cumulative.

FIG. 10. An example of a wave of Ca^{2+} release in a KCl-activated *Chaetopterus* egg. (a–f) The photons accumulated after successive 2-sec intervals. These data were obtained using the Hamamatsu system, so the photons shown in successive panels are cumulative. This wave moved from the top to the bottom. The wave took approximately 6 sec to traverse the egg.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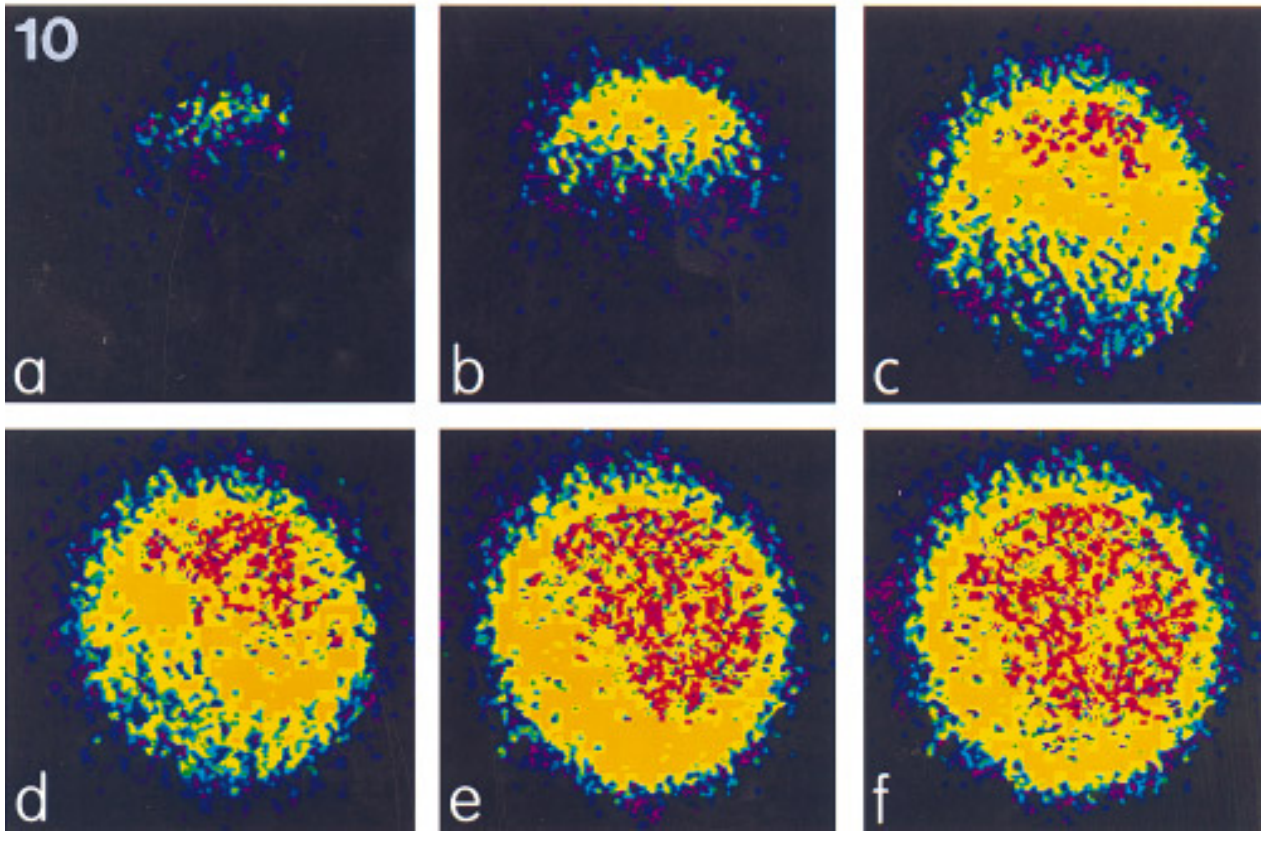
10
a
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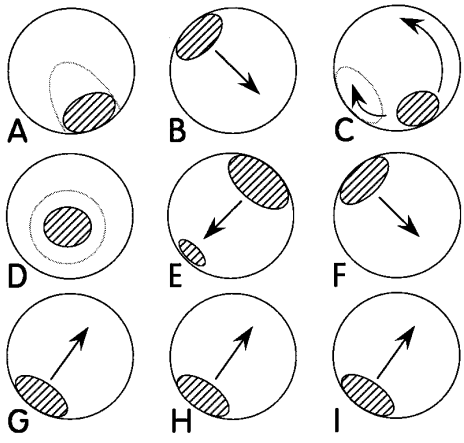

FIG. 5. Origin and direction of travel for the resolved transients shown in Fig. 2. The letter by each refers to the letter of the transient indicated in Fig. 2. Hatched areas in each represent the origin of the transient. Arrows indicate the direction of propagation of waves. Straight arrows indicate waves that appeared to propagate across the entire egg; curved arrows represent waves that were definitely restricted to the cortex. The lighter lines in A and D represent the largest extent of the transients. These transients did not completely propagate through the egg. The lighter oval in C represents the final area in which the wave ended up and lingered. The smaller hatched area in E represents the position of a nonpropagating transient that began at the same time as the wave indicated by the larger hatched area.

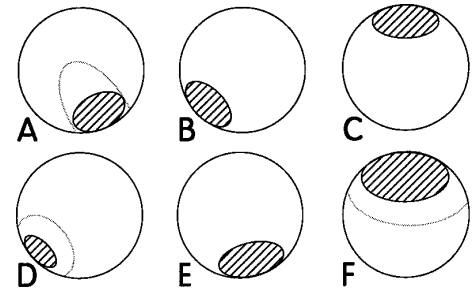

FIG. 6. Diagrammatic representations of the first pulses from each of the eggs in Table 1. Some, such as those in A, D, and F, appeared to propagate part of the way through the egg. Hatched areas in each represent the origin of the transient. The lighter lines in A, D, and F represent the largest extent of the transients. None of these transients propagated across the entire egg.

known that would result in simultaneous Ca^{2+} release throughout the cytoplasm of such a large cell (Jaffe, 1993). However, despite the fact that no mechanism currently known can account for it, global, synchronous Ca^{2+} release has recently been reliably reported in spontaneously maturing mouse oocytes (Carroll *et al.*, 1994). We are currently developing the technology for 3-D luminescent imaging by collecting photons from different optical sections. We intend to reexamine *Chaetopterus* fertilization when this equipment and software become available.

Are repetitive Ca^{2+} waves and pulses such as those we report here characteristic of protostome fertilization in general? Although the available data are limited, we can provide a tentative answer in the affirmative. Ca^{2+} transients have also been measured in four bivalve mollusc species at fertilization. In each, repetitive Ca^{2+} transients were detected (Deguchi and Osanai, 1994). Unfortunately, the spatial distribution of only the first transient was investigated and only in one of the species. The Ca^{2+} increase during

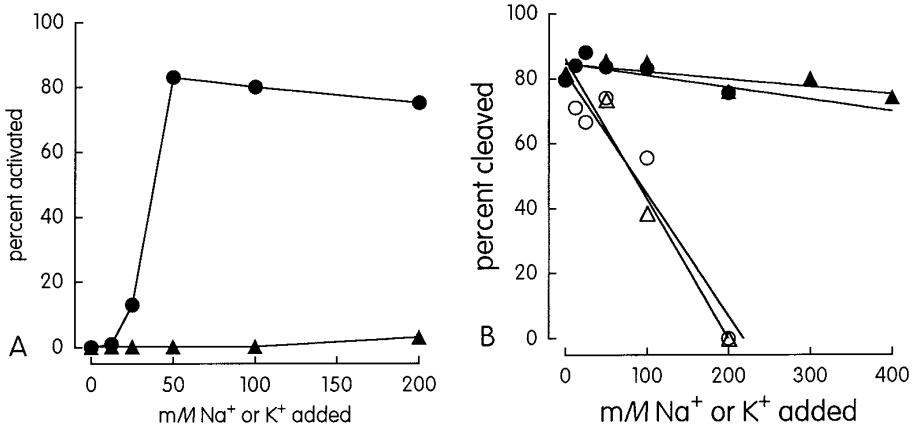

FIG. 7. Effect of elevated extracellular K^+ and Na^+ on activation and cleavage of *Chaetopterus* eggs. (A) Dose-dependent activation of eggs by excess extracellular K^+ as evidenced by the initiation of ameboid activity (''pseudocleavage'') characteristic of parthenogenetically activated eggs in this species. Circles represent excess K^+ added; triangles represent excess Na^+ added. (B) Dose-dependent inhibition of cleavage of fertilized *Chaetopterus* eggs by exposure to excess extracellular K^+ and Na^+. Open symbols indicate continuous exposure to excess Na^+ or K^+; solid symbols indicate a 20-min exposure to excess Na^+ or K^+. Circles represent excess K^+ added; triangles represent excess Na^+ added.

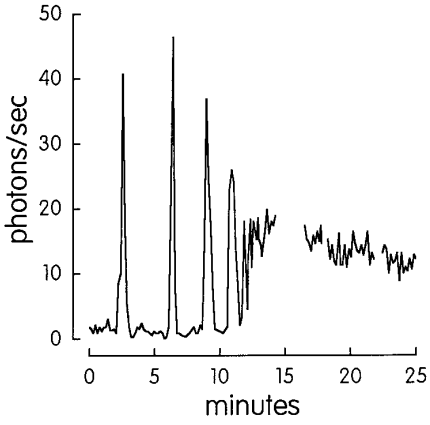

FIG. 8. Sequence of Ca^{2+} transients in a *Chaetopterus* egg microinjected with semisynthetic *f*-recombinant aequorin and then activated with excess K^+. Photon counts/sec were averaged over successive 9.6-sec intervals and plotted. This egg underwent four Ca^{2+} transients over a 10-min period after fertilization, after which the Ca^{2+} level remained elevated for at least an additional 10 min. The breaks in the trace at 15, 18, and 22 min result from switching termporarily to bright-field microscopy to examine the eggs.

the first transient was reported to be uniform throughout the cytosol. The time required for this transient to reach its peak was less than 5 sec, or faster than the transit time we have observed for waves in *Chaetopterus*. Our results agree that the first transient is generally not a wave, but they differ in that the first transient we observed was never global. As noted above, it is unclear how Ca^{2+} could be released simultaneously throughout a cell.

Mammalian eggs also undergo repeated Ca^{2+} transients (Ben-Yosef *et al.*, 1993; Cheek *et al.*, 1993; Miyazaki *et al.*, 1993), at least superficially similar to those in *Chaetopterus* and bivalves. Some of the transients in mammalian eggs are waves, but it is unclear whether they all are. Neither is it known whether the multiple transients are initiated at different points on the egg surface, such as we describe here.

Eggs of organisms as phylogenetically diverse as annelids, molluscs, echinoderms, ascidians, and vertebrates all release stored intracellular Ca^{2+} in response to fertilization and exhibit elevated cytosolic Ca^{2+} over an extended period after fertilization. This suggests that such activation mechanisms are characteristic of all animal eggs.

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